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NOTES

Construction and Characterization of a Human T-Cell Lymphotropic Virus Type 3 Infectious Molecular Clone▽

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We and others have uncovered the existence of human T-cell lymphotropic virus type 3 (HTLV-3). We have now generated an HTLV-3 proviral clone. We established that *gag*, *env*, *pol*, *pro*, and *tax*/*rex* as well as minus-strand mRNAs are present in cells transfected with the HTLV-3 clone. HTLV-3 p24^{gag} protein is detected in the cell culture supernatant. Transfection of 293T-long terminal repeat (LTR)-green fluorescent protein (GFP) cells with the HTLV-3 clone promotes formation of syncytia, a hallmark of Env expression, together with the appearance of fluorescent cells, demonstrating that Tax is expressed. Viral particles are visible by electron microscopy. These particles are infectious, as demonstrated by infection experiments with purified virions.

Phylogenetic analyses have provided supporting evidence that multiple episodes of interspecies virus transmission have occurred between nonhuman primates and humans (9, 16). Examples are human T-lymphotropic viruses (HTLVs) and their related simian counterparts (STLVs) that all belong to the primate T-cell lymphotropic viruses (8, 10, 23). STLV type 3 (STLV-3) was isolated in 1994 from a captive baboon (*Papio hamadryas*) (7). It is now well established that STLV-3 strains are widespread in a number of simian species living in West, East, and Central Africa (5, 7, 12–15, 18–22). HTLV type 3 (HTLV-3), the human counterpart of STLV-3, was discovered in 2005 by our laboratory and another (3, 17, 24), and a third strain has been described more recently (1). We sequenced the 8,553-bp genome of an HTLV-3 strain (HTLV-3_{Pyl43}) (2) and showed that it is very similar to that of STLV-3_{CTO604}, a simian strain from Cameroon (14). However, sequence comparisons also revealed that the HTLV-3_{Pyl43} genome is shorter than the STLV-3 sequences, due to a 366-bp deletion in the pX proximal region. Of note, this deletion does not affect *tax*, *rex*, or *env* sequences (2).

Using a PCR-based strategy, we recently developed the first infectious STLV-3 molecular clone (4). Here, we have employed the same strategy to construct an HTLV-3 molecular clone, with HTLV-3_{Pyl43} DNA as a source of proviral material. In a first series of experiments, we generated the full-length 8,853-bp HTLV-3_{Pyl43} provirus by PCR amplification of 20 overlapping fragments as previously described (4). The proviral sequence was ligated into the $SV2_{neo}$ plasmid between the EcoRI and HpaI restriction sites (Fig. 1A, top panel). Clones were then screened by digesting the plasmids with EcoRI plus BamHI plus ScaI or with PstI or XbaI (Fig. 1A, top, middle, and bottom panels). Two clones ($SV2_{Pyl43\ cl9}$ and $SV2_{Pyl43\ cl26}$) displayed the expected restriction digestion pattern (Fig. 1B, lanes 2 to 3, 5 to 6, and 8 and 9), indicating that these plasmids contained the full-length HTLV-3_{Pyl43} provirus. Full sequence analysis was also performed on both clones and demonstrated that neither mutations nor deletions that would alter the different viral protein sequences had been introduced in the HTLV-3_{Pyl43} provirus during the cloning process (data not shown).

We then determined whether our molecular clone was capable of directing mRNA synthesis in cell culture. To this end, $SV2_{Pyl43\ cl9}$ and $SV2_{Pyl43\ cl26}$ plasmids were transfected into 293T cells as described previously (4). After 2 days, total RNA was extracted and treated twice with DNase I. Reverse transcription-PCR experiments were then performed to detect different mRNA viral species—*gag*, *pro*, and *pol* (nonspliced); *env* (singly spliced); and *tax*/*rex* (doubly spliced)—as well as a putative mRNA transcribed from the minus strand of the genome. This mRNA could be translated into a protein that we tentatively named AEP (antisense-encoded protein).

Total RNA (0.5 μg) was used as a matrix for reverse transcriptase PCR (RT-PCR) with the OneStep RT-PCR kit (Qiagen). PCR was performed using the following primer pairs: for Gag, LTR681s (GGAGAAAGCAAACAGGTGGGGG) and GAG1119as (GTGGGGGTGAAGGACAGGGAGG) (459-bp RT-PCR product); for Pro, Pro2016se (5′-AGGACTAACCTC CCCCCGGACC-3′) and Pro2412as (5′-GAGAACACTTGA GGGTTGGTCAGC-3′) (397-bp RT-PCR product); for Pol, Pol4029s (5′-CCATCCACCCAGTGTGACCTACAC-3′) and Pol4633as (5′-GGGTTGTAGGGAACATGGGTTGAAT-3′)

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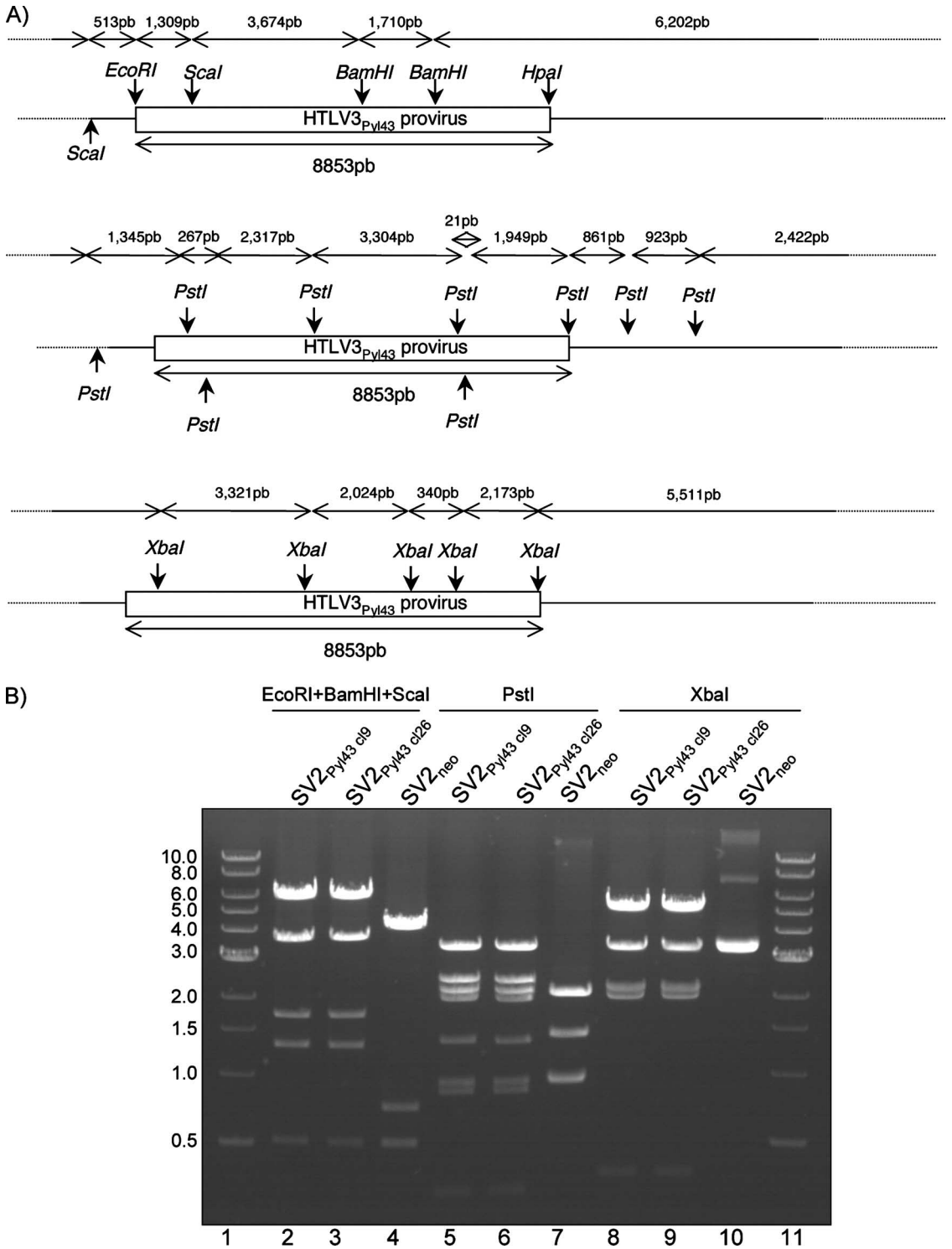


FIG. 1. Construction of the $SV2_{Pyl43}$ clone and expression of the viral mRNAs in vivo. (A) Restriction map of the full-length HTLV-3_{Pyl43} genome inserted into the $SV2_{neo}$ plasmid. (B) Lanes 1 and 11, 1-kb DNA ladder; lanes 2, 3, and 4, restriction profiles of $SV2_{Pyl43\ cl9}$, $SV2_{Pyl43\ cl26}$, and $SV2_{neo}$ backbone plasmids digested with EcoRI plus ScaI plus BamHI and run on a 0.7% agarose gel; lanes 5, 6, and 7, plasmids digested with PstI; lanes 8, 9, and 10, plasmids digested with XbaI. pb, paired bases.

(605-bp RT-PCR product);for Env, LTR111s (CCAAGGCTC TGACGTCTCTCCCTAC) and Env5117as (TGGGATTGCC AAAAGAGGAAGGG) (516-bp RT-PCR product); for Tax, 602LTR and 602MVB Rex (14) (424-bp RT-PCR product); and for AEP, Pyl43-AEPs (5′-GGAGGCTCCAACCTCAGG-3′) and Pyl43-AEPas (5′-ACTCCGCCACTTCCTGTAG-3′) (274-bp RT-PCR product).

As seen in Fig. 2A (lanes 4 and 5, top, middle, and bottom

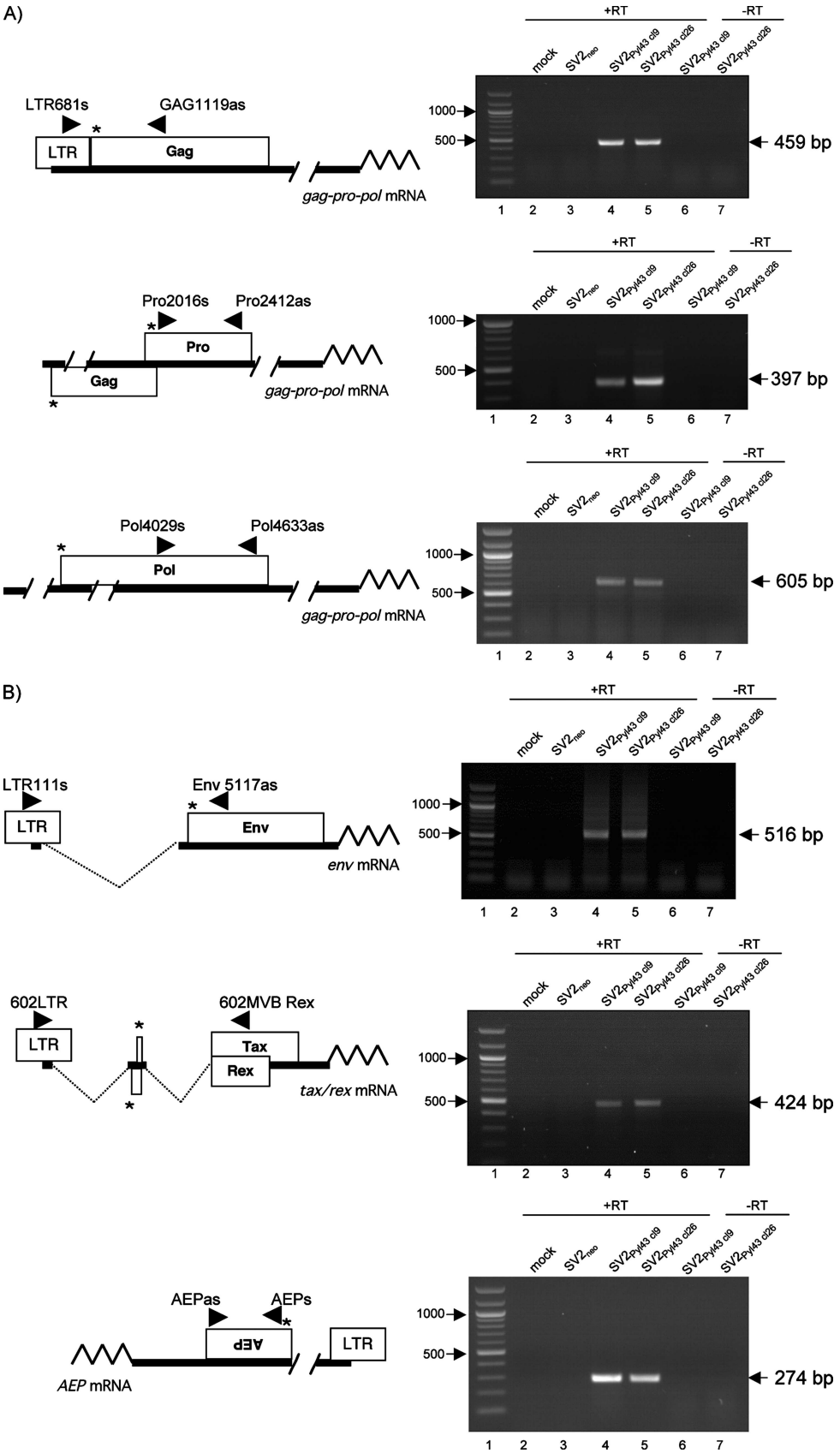
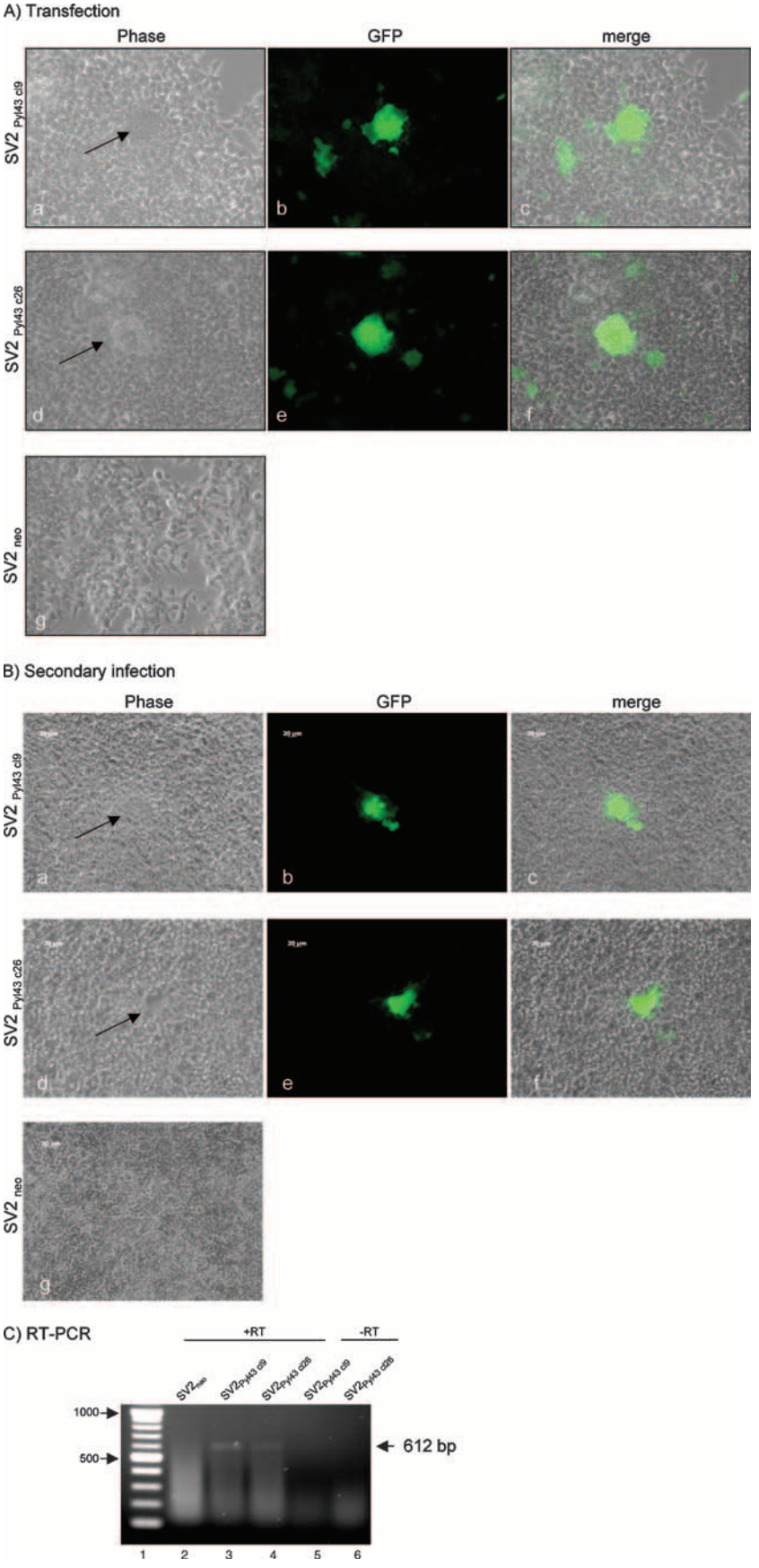


FIG. 2. HTLV-3_{Pyl43} provirus is transcribed in vitro. (A and B) RT-PCR analysis of $SV2_{Pyl43}$ viral RNAs. Total RNA was extracted from 293T cells transfected with $SV2_{Pyl43\ cl9}$, $SV2_{Pyl43\ cl26}$, and $SV2_{neo}$ plasmids or mock transfected. (A) Top, *gag*; middle, *pro*; and bottom, *pol*. (B) Top, *env*; middle, *tax*/*rex*; bottom, AEP. (A and B) Lanes 1, 100-bp DNA ladder; lane 2, mock-transfected 293T cells; lane 3, RNA from $SV2_{neo}$ (backbone vector)-transfected cells; lanes 4 to 7, RNA from cells transfected with $SV2_{Pyl43\ cl9}$ and $SV2_{Pyl43\ cl26}$ plasmids in the presence (lanes 4 and 5) or absence (lanes 6 and 7) of RT. *, ATG.


A) Transfection
Phase
GFP
merge
SV2 Pyl43 cl9
SV2 Pyl43 c26
SV2 neo
a
b
c
d
e
f
g
B) Secondary infection
Phase
GFP
merge
SV2 Pyl43 cl9
SV2 Pyl43 c26
SV2 neo
a
b
c
d
e
f
g
C) RT-PCR
+RT
-RT
SV2neo
SV2Pyl43 cl9
SV2Pyl43 c26
SV2Pyl43 cl9
SV2Pyl43 c26
1000
500
612 bp
1
2
3
4
5
6

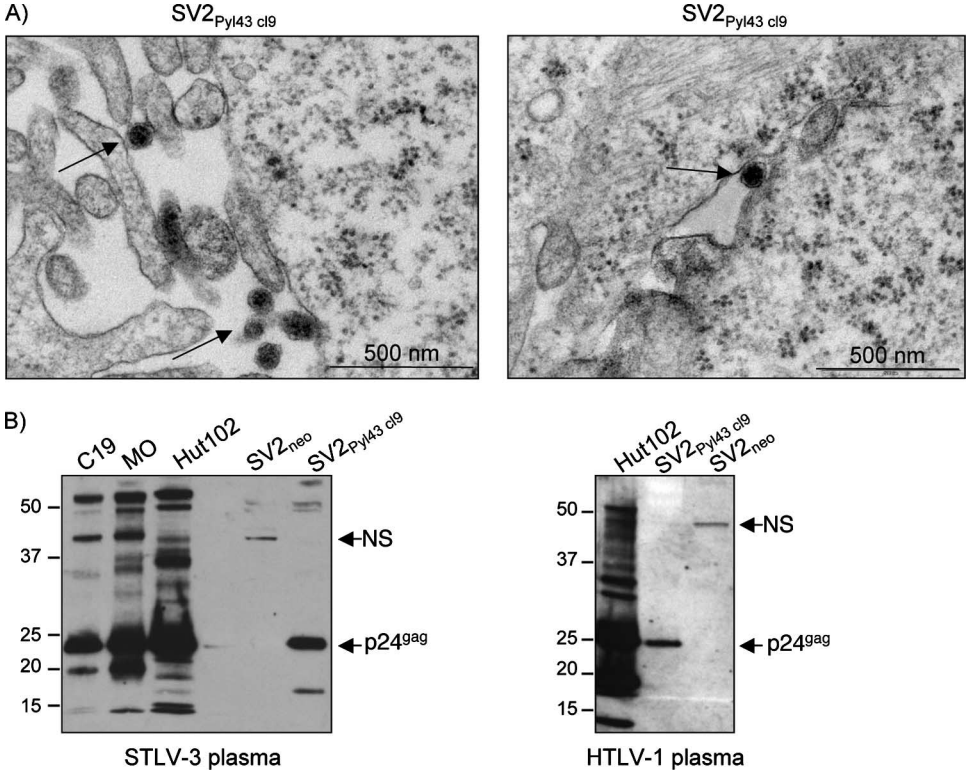


FIG. 4. (A) Electron micrograph showing HTLV-3_{Pyl43} particles in $SV2_{Pyl43\ cl9}$-transfected cells. (B) Expression of HTLV-3 $p24^{gag}$ protein. 293T cells were transfected with $SV2_{Pyl43\ cl9}$ or $SV2_{neo}$ plasmid. Growth medium was collected. After lysis, viral proteins were transferred to a membrane and incubated with plasma obtained from an STLV-3-infected monkey (PPA-F8) (left) or plasma obtained from an HTLV-1-infected TSP/HAM patient (PH1378) (right). These Western blots are representative of three different experiments.

panels), an HTLV-3-specific band corresponding to different part of the *gag pro pol* transcript was present only in extracts obtained from $SV2_{Pyl43}$-transfected cells. The absence of a PCR product when RT was omitted demonstrates the lack of DNA carryover in the RNA preparation (Fig. 2A, lanes 6 and 7, top, middle, and bottom panels). *env* and *tax*/*rex* transcripts were also present in these cell extracts (Fig. 2B, top and middle panels). Finally, we also demonstrated that, as in HTLV-1, the HTLV-3 minus strand is transcribed (Fig. 2B, bottom panel). Whether the protein that is translated from this mRNA is functionally related to HBZ remains to be determined. Next, 293T-long terminal repeat (LTR)-green fluorescent protein (GFP) indicator cells (6) were transfected with either the $SV2_{Pyl43\ cl9}$ or $SV2_{Pyl43\ cl26}$ plasmid.

The appearance of syncytia is linked to the interaction of the viral envelope on the surface of the infected cells with the viral receptors that are present on the surface of adjacent cells. Forty-eight hours posttransfection, cell culture medium was removed. Cells were washed with phosphate-buffered saline and fixed, and pictures were taken with a Zeiss Axioplan-Axiocam-apotome system (Fig. 3A). As expected, syncytium formation was observed after transfection of $SV2_{Pyl43\ cl9}$ or $SV2_{Pyl43\ cl26}$ plasmid in the 293T-LTR-GFP cells demonstrating HTLV-3 envelope expression (Fig. 3A, panels a and d). These syncytia were GFP positive (Fig. 3A, panels b and c and e and f), therefore establishing that the Tax protein was expressed and able to transactivate the viral promoter in these cells. GFP signal and syncytia were not visible in cells transfected with the empty backbone vector (Fig. 3A, panel g).

To determine whether $SV2_{Pyl43}$-transfected cells produce infectious particles, cell culture supernatant was collected from $SV2_{Pyl43\ cl9}$- or$SV2_{Pyl43\ cl26}$-transfected cells, purified, and added to 293T-LTR-GFP indicator cells as previously described (4) (Fig. 3B). After several days of culture, a number of GFP-positive syncytia were reproducibly observed (Fig. 3B, panels a and d). These syncytia were GFP positive (panels b and c and e and f). As a control, we did not observe any syncytia when 293T-LTR-GFP cells were put in contact with

FIG. 3. Viral envelope and Tax expression. (A) 293T-LTR-GFP indicator cells were transiently transfected with $SV2_{Pyl43\ cl9}$ (a to c), $SV2_{Pyl43\ cl26}$ (d to f), or $SV2_{neo}$ (g) backbone vector. The images shown are representative of three different experiments. (B) $SV2_{Pyl43}$ viral particles are infectious. Growth medium was collected from cells transfected with $SV2_{Pyl43\ cl9}$ (a to c), $SV2_{Pyl43\ cl26}$ (d to f), or SV2neo (g) molecular clones and added to 293T-LTR-GFP indicator cells as previously described (4). The images are representative of three different experiments. (C) RT-PCR analysis of $SV2_{Pyl43}$ viral RNA extracted from cells infected with purified HTLV-3 viral particles. Lane 1, 100-bp DNA ladder; lane 2, RNA from $SV2_{neo}$ (backbone vector)-transfected cells; lanes 3 to 6, RNA from cells transfected with $SV2_{Pyl43\ cl9}$ and $SV2_{Pyl43\ cl26}$ plasmids in the presence (lanes 3 and 4) or absence (lanes 5 and 6) of RT.

supernatant from cells transfected with the backbone vector (Fig. 3B, panel g). We also performed RT-PCR on the RNA extracted from cells infected with cell-free virus. RNA was extracted and reverse transcribed as described above. PCR was then performed with primers located within the *gag* open reading frame: LTR681s (5′-GGAGAAAGCAAACAGGTGGGGG-3′) and Gag1293as (5′-TCATGGAGATCTTTAGCTGTGGGG-3′ (PCR product of 612 bp).

This allowed us to demonstrate that *gag pro pol* mRNA was present in these cells (Fig. 3C). Altogether, these results demonstrate that the purified HTLV-3_{Pyl43} particles are infectious.

We also wanted to observe viral particles. Forty-eight hours posttransfection of 293T with the molecular clone, cell culture medium was removed and the cells were washed with phosphate-buffered saline and fixed for ultrastructural analyses as previously described (4). Viral particles were then detected in $SV2_{Pyl43\ cl9}$-transfected cells by electron microscopy (Fig. 4A) but not in the cells transfected with the backbone vector (data not shown). The size of these particles is roughly similar to that of STLV-3 particles (4).

Finally, the supernatant of 293T cells transfected with $SV2_{Pyl43\ cl9}$ was analyzed (Fig. 4B). Growth medium was collected, clarified by low-speed centrifugation, and filtrated. Virus was then layered on a 20% glycerol gradient and centrifuged. The pellet was resuspended in lysis buffer. Each sample was resolved by electrophoresis on a 10% *N*,*N*-methylenebisacrylamide–Tris gel. As controls, the supernatant from HTLV-1-infected (Hut102) and/or HTLV-2-infected (C19, MO) cell cultures was also tested. The membrane was incubated using STLV-3 plasma (Fig. 4B, left panel) or HTLV-1 plasma (right panel). With both sera, a band corresponding to the HTLV-3 $p24^{gag}$ protein was observed in the supernatant obtained from $SV2_{Pyl43\ cl9}$-transfected cells, but not in the protein extracts from $SV2_{neo}$-transfected cells. Interestingly, the STLV-3 plasma also allowed the detection of the HTLV-1 and HTLV-2 $p24^{gag}$ protein (Fig. 4B, left panel).

Altogether, our data demonstrate that the $SV2_{Pyl43\ cl9}$ molecular clone is functional and produces infectious viral particles. Comparison of the viral life cycles of both STLV-3 and HTLV-3 in a rabbit model (11) will now allow us to ascertain whether the 366-bp deletion impacts either viral infectivity or replication in vivo. Finally, given the fact that an HTLV-3-infected cell line is not yet available, this clone will be a unique and powerful tool that will allow us to investigate HTLV-3 protein expression and viral pathogenesis in vivo.

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